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DEVELOPMENT OF A THEORETICAL MODEL TO ASSESS THE HEPATOCARCINOGENIC POTENTIAL OF CHEMICALS USING STRUCTURE-ACTIVITY RELATIONSHIPS AND THE RAT HEPATOCYTE ASSAY

Final Report

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Atomic charges for all atoms in the DNT molecules were calculated by the semi-empirical MNDO molecular orbital method. A linear correlation was developed when the log EC<sub>50</sub> concentration for LDH release was plotted against C-atomic charge for reactive nitro groups. This correlation was used to predict the EC<sub>50</sub> values for untested nitroaromatic compounds.

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## I. EXECUTIVE SUMMARY

Rat hepatocyte (liver cell) suspensions were used to investigate structure-activity relationships in six isomeric dinitrotoluenes. Biological parameters that could be correlated with structure were lactate dehydrogenase (LDH) release and inhibition of protein synthesis. For each biological parameter, the DNT isomers that exhibited the greatest cytotoxic potency had their nitro group oriented ortho or para to each other (2,5-DNT, 2,3-DNT, 3,4-DNT) and the DNT isomers that were the least toxic had the meta orientation (2,4-DNT, 2,6-DNT, 3,5-DNT). High-performance liquid chromatographic profiles of the reaction medium indicated that reduction was the major metabolic pathway for each isomer except in the case of 2,6-DNT, where oxidation of the methyl group was dominant. The similarity in the slopes of the concentration response to the DNT isomers suggests the possibility of a common intermediate as the causative agent for cytotoxicity. The atomic charges on carbon atoms bearing nitro groups, as computed by quantum mechanical methods, were used to correlate cytotoxic potential with molecular structure. Based on this finding, the cytotoxic potentials of untested nitroaromatic compounds were projected.

## II. FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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### III. INTRODUCTION

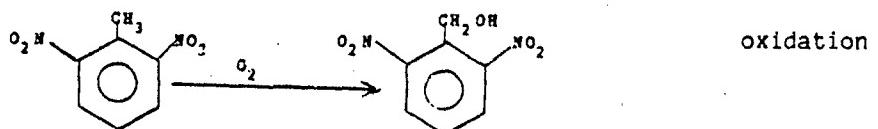
Nitroaromatic compounds represent an important class of compounds used in the manufacture of explosives, dyes, drugs, and pesticides. Dinitrotoluenes (DNTs), which are important chemical intermediates, are also found in the wastewaters from trinitrotoluene (TNT) manufacturing facilities (Spanggord et al., 1982). 2,6-DNT, one of the isomers of DNT, has been shown to be a carcinogen in laboratory animals (Popp and Leonard, 1983). On the other hand, 2,4-DNT showed no evidence of being carcinogenic in rats or mice in a National Cancer Institute study (1978), but the technical grade 2,4-DNT (containing 76% 2,4-DNT, 19% 2,6-DNT, 2.4% 3,4-DNT, 1.5% 2,3-DNT, 0.65% 2,5-DNT, and 0.04% 3,5-DNT) was shown to be carcinogenic in rats (Ellis et al., 1979; CIIT, 1982). Although it has been suggested that 2,6-DNT is responsible for the carcinogenicity of the technical grade material, it is by no means clear from available experimental evidence that 2,6-DNT is the only--or even the most potent--carcinogen in the mixture, and what molecular properties influence carcinogenic potential. In the studies showing the carcinogenicity of 2,6-DNT, the primary organ involved was the liver. This suggested that hepatocytes might be used to study specific molecular properties in isomeric DNTs from which structure-activity relationships could be derived to project carcinogenic potential. The studies performed herein were designed with this objective in mind.

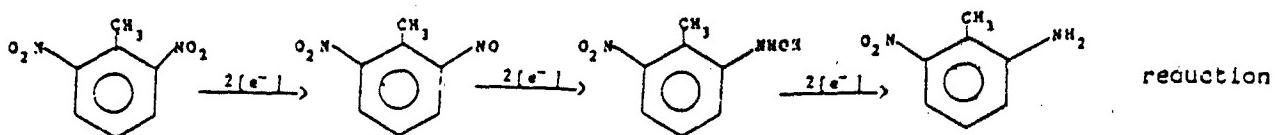
#### IV. BACKGROUND

It is widely believed that tumorigenicity is initiated by the interaction of chemical carcinogens with cellular macromolecules such as DNA. In hepatocarcinogenesis, the initial step is followed by a promotion stage in which the chemical can induce replication of the initiated cells and growth of the focal lesions into tumors (Farber, 1984). Chemicals can act either as initiators, promoters, or complete carcinogens in the development of tumorous cells (Pitot and Sirica, 1980). Using hepatic initiation-promotion protocols, Leonard et al. (1983) found that purified 2,6-DNT and technical-grade 2,4-DNT could initiate rat hepatocytes by increasing  $\gamma$ -glutamyltranspeptidase (GGT<sup>+</sup>) foci; the five remaining purified DNT isomers studied possessed negligible initiating activity. The initiating activity for technical grade 2,4-DNT or 2,6-DNT was weak. In a protocol for tumor promotion with technical grade 2,4-DNT, purified 2,4-DNT, and 2,6-DNT, all three exhibited promoting activity by inducing enzyme-altered foci with diethylnitrosamine (Popp and Leonard, 1982). Promotion studies with the other isomers were not conducted, presumably because of limited supplies. A much greater number of foci were found in the promotion studies than in the initiation studies for the corresponding DNTs. In addition, Bermudez et al. (1979) found no induction of unscheduled DNA syntheses (UDS) in primary rat hepatocytes when the cultures were treated with any of the six DNT isomers. This may suggest that cecal microflora are important contributors to the mechanism of tumor induction by DNTs. This was later inferred by Mirsalis and Butterworth (1982), who found 2,6-DNT to be a potent inducer of UDS and 2,4-DNT a weak inducer in rat hepatocytes after *in vivo* treatment. Most recently, Dixit et al. (1986) reported that cecal microflora-generated metabolites from 2,4-and 2,6-DNT produced approximately the same degree of binding to DNA in 24-hr hepatocyte cultures. The sum of these studies suggests that the promoting activity of the DNTs is very important in the ultimate development of tumors and that the metabolites derived from microflora, hepatocytes, or both are the responsible agents.

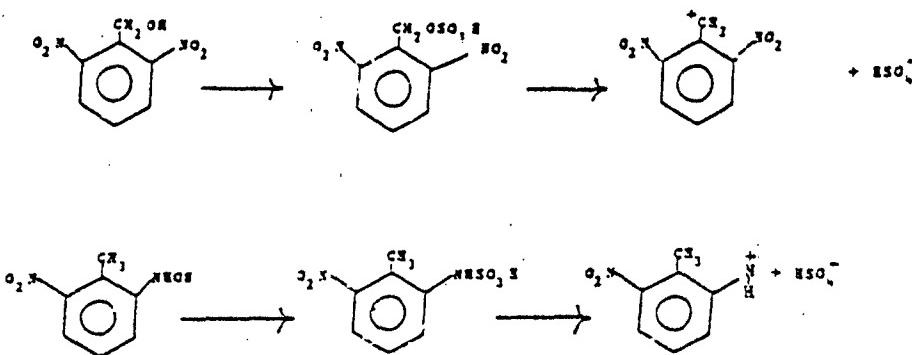
The biotransformation of DNT is complex; both oxidative and reductive transformations in liver preparations have been observed (Bond and Rickert, 1981). These transformations, coupled with the reductive transformations caused by microflora in the intestine, tend to obscure the identification of the ultimate carcinogenic metabolite(s) of DNT.

The reductive and oxidative transformations of DNTs, using 2,6-DNT as an example, are shown below.





A proposed mechanism for tumorigenicity involves the generation of an electrophilic species from one of the above metabolites. This can be accomplished by sulfation of a hydroxyl group followed by cleavage as shown below.



The electrophiles then covalently bind to cellular macromolecules. Recurring or transient cytotoxicity, which can be produced as a result of such action, is thought to play an integral role in hepatocarcinogenesis by inducing selective replication of initiated cells (Farber, 1984). If one of the proposed pathways is correct, there should be an effect of the position of a neighboring nitro group on cellular damage as determined by lactate dehydrogenase (LDH) release or through the inhibition of protein synthesis, commonly used indicators of cytotoxicity (Goethals et al., 1984). Also, the importance of cecal microflora--as measured by biological effects in in vivo or in vitro studies--suggests that reductive metabolism is a key step. Further, the complete reduction of a nitro group to the amine is a six-electron process that generates three intermediate free radicals. The effect of these radicals in instituting cellular damage might be detected by evidence of lipid peroxidation occurring within the cell.

On this reasoning, the three parameters--LDH release, protein syntheses, and lipid peroxidation--were evaluated in isolated rat hepatocyte systems for each of the six DNT isomers in the absence of and following treatment with cecal microflora. Isolated hepatocyte systems are becoming increasingly useful for a variety of toxicological applications, including structure-activity correlations where quantities of test compounds are insufficient for in vivo testing (Rauckman and Padilla, *in press*). The chromatographic profile of metabolites was then used to identify major metabolism routes. The combined data were used to develop structure-activity relationships based on molecular properties.

## V. METHODS

### Animals

Male Sprague-Dawley rats (320 to 410 g) were obtained from Simonsen Laboratories (Gilroy, CA). The rats were kept in clear plastic cages, maintained under a standard light:dark (12L:12D) cycle for at least 2 weeks before use, and allowed feed (Purina Lab Chow) and water ad libitum.

### Hepatocyte Isolation and Culture

Hepatocytes were isolated by whole liver perfusion as described by Green et al. (1983). The animals were anesthetized with pentobarbital (65 mg/kg administered ip), and the abdomen was shaved and washed with 70% ethanol. The liver was exposed via a midline abdominal incision, and the gut was pushed to the side to expose the portal vein. The vein was cannulated and perfused with an EGTA-containing buffer at a flow rate of 35 ml/min to clear the blood. The vena cava was cut and the liver was carefully dissected out. After 20 min of perfusion, the liver was perfused with a  $\text{Ca}^{2+}$ -supplemented collagenase buffer (40 U of collagenase/ml) until the tissue was digested (15 to 20 min). Individual cells were freed by gentle disruption of the tissue with a fork and filtered through gauze. The hepatocyte suspension was separated from cell debris by differential centrifugation at 50 x g and resuspended in fresh EGTA perfusate three times. The cell count and viability of the hepatocyte stock suspension were determined by trypan blue exclusion (Green et al., 1983); the viability averaged  $90.7 \pm 3.2\%$  for the preparations used in the experiments.

After isolation, the hepatocytes were diluted to  $6 \times 10^6$  viable cells/ml in hormone-supplemented Waymouth's 752/1 culture medium (Green et al., '83), based on the formulation of Decad et al. (1977). For experiments, 1.0 ml of hepatocytes was added to 25-ml Erlenmeyer flasks each containing 3.0 ml of culture medium. Treated flasks also contained the DNT compound at the desired final concentration, introduced into the culture medium in DMSO solution. The DMSO content in the flasks did not exceed 1%. Each flask was immediately gassed with a 95% air:CO<sub>2</sub> atmosphere for 3 sec and placed in a shaking water bath at 37°C (65-70 oscillations/min). All experiments included a negative control (DMSO only) run under identical conditions except for the omission of DNT. Each measurement was made on duplicate or triplicate flasks, depending on the number of variables being evaluated and cells available for the experiment.

Normally, incubations proceeded for 4 hr before the experiment was terminated. Cytotoxicity parameters assessed were LDH release from the cells, rate of incorporation of L-valine into cell protein (protein synthesis), and malondialdehyde (MDA) equivalents and/or ethane evolution (lipid peroxidation). For the determination of LDH release, two 0.10-ml aliquots were removed from the flask at 4 hr. One sample was mixed with 0.10 ml of 1% Triton X-100 to lyse the cells for analysis of total LDH content. Both samples were then centrifuged at 1200 x g for 3 min to pellet cells and debris. The supernatants were removed with Pasteur pipettes, transferred to test tubes, and refrigerated until analysis (within 24 hr) (Story et al., 1983). For lipid

peroxidation assessed by the thiobarbituric acid (TBA) reaction, 0.25-ml aliquots were transferred to screw-top test tubes, 0.50 ml of 15% trichloroacetic acid (TCA) was added immediately, and the samples were frozen for later analysis. For protein syntheses, [<sup>14</sup>C]valine was added to the flasks after 3 hr of incubation. Aliquots (0.50 ml each) of the cell suspension were transferred to test tubes at 4 hr and the protein was precipitated with 10% perchloric acid (PCA).

#### Incubation with Cecal Microflora

Cecal microflora were isolated by the following method. Male Sprague-Dawley rats (130 to 180 g) inoculated at birth with Charles River altered Schraedaler flora (CRASF) were anesthetized with pentobarbital (65 mg/kg body weight administered ip). The abdomen was washed with 70% ethanol and the peritoneal cavity was opened to expose the cecum. Hemostats were attached to each end of the cecum, which was then excised and opened. The contents were transferred into a preweighed, 50-ml centrifuge tube that contained 10 ml of potassium phosphate buffer, pH 7.4, and that had been gassed for 30 to 60 min with nitrogen (>99%) to remove all traces of oxygen. The tube was vortexed to mix the contents thoroughly and again weighed. The cecum weighed 4.17 g and 6.01 g, respectively, in the two experiments conducted.

For metabolism of DNTs cecal contents were centrifuged at 200 x g for 2 min to remove large particles. Next 250  $\mu$ l of the supernatant were used to inoculate 5 ml of potassium phosphate buffer, pH 7.4, in a glass side-arm flask that had been pregassed with nitrogen for 60 min. The incubation was initiated by adding 50  $\mu$ l of the appropriate chemical stock to the side-arm flask and mixing it with the contents in the main flask to give final chemical concentrations of 2.0 mM, 1.0 mM, and 1.5 mM solutions of 2,4-DNT, 2,5-DNT, and 2,6-DNT, the approximate solubility limit for each in the medium, respectively. After incubation at 37°C in a shaking water bath for 60 min, air was admitted to each flask and the contents were filtered through 0.45- $\mu$  filters. As a control, identical samples were tested for each condition in the absence of cecal microflora. The filtrates were mixed with equal volumes of culture medium plus hepatocytes and incubated as described for cytotoxicity studies.

#### Analytical Methods

##### LDH Release

Aliquots for LDH determinations were analyzed on a Gemini minicentrifugal analyzer (Electro-Nucleonics, Inc., Fairfield NJ), using Beckman Dri-Stat reagents (Beckman Instruments, Carlsbad, CA). The technique involves the measurement of NADH formation from NAD<sup>+</sup> through the increase in absorbance at 340 nm. One unit of enzyme activity is the amount of enzyme that converts 1  $\mu$ mol of substrate to product per min per liter of sample, concurrent with the reduction of a corresponding amount of NAD<sup>+</sup>. Percent LDH release is the net release of enzyme from the cells to the supernatant induced by the chemical divided by the total enzyme content of the flask after background release (control cells with no chemical present) is subtracted from each quantity (Story et al., 1983). Total enzyme content in the flask is the activity measured in Triton X100-treated aliquots.

### Protein Synthesis

Protein synthesis rates were determined by measuring the incorporation of [<sup>14</sup>C]valine (0.50 mCi/mmol) into acid-precipitable material after a 60-min incubation in the culture medium as described above. The PCA precipitates were thawed and centrifuged at 1200 x g for 5 min. The precipitated protein was washed three times with 3.0 ml of 2% PCA by vortexing the suspension and pelletting the precipitate by centrifugation at 1200 x g for 5 min. The precipitate was then dissolved in 0.50 ml of 0.3 N NaOH, transferred to scintillation vials with 2.0 ml of distilled water, and mixed with 10 ml of Neutralizer scintillation fluid (Research Products International, Mount Prospect, IL). The radioactivity was determined by liquid scintillation counting. Data are expressed as  $\mu$ mol of [<sup>14</sup>C]valine incorporated/min/mg protein and converted to percent of control activity. Protein content of the incubation was determined by the method of Bradford (1976) on aliquots from the same tubes.

### Thiobarbituric Acid (TBA) Reactants

TBA solution (0.67%: prepared by mixing 2 parts of 1% TBA neutralized in aqueous NaOH plus 1 part of 0.5M citrate buffer, pH 2.0, was added to the thawed aliquots. The mixture was heated at 92°C for 30 min in a boiling-water bath. After cooling to room temperature, the sample was centrifuged at 2500 rpm on a bench-top centrifuge for 10 min. The supernatant was then transferred to a cuvette and the absorbance at 530 nm was measured and recorded with a Gilford Model 220 spectrophotometer (Gilford Instrument Laboratories, Oberlin OH). The reading was converted to nmoles malondialdehyde MDA based on a standard curve using authentic samples.

### Ethane Evolution

Ethane evolution is a sensitive and reliable alternate indicator for lipid peroxidation (Kappus, 1985; Tyson and Green, in press). When ethane evolution was quantitated, the experimental conditions were modified to maximize sensitivity, as follows. The total cell content was doubled to  $12 \times 10^6$  viable cells/flask by increasing the aqueous phase to 8.0 ml, thereby reducing the headspace from 16 to 12 ml. The incubation period was shortened to 2 hr because of more limited O<sub>2</sub> available in the flask under these conditions (Smith et al., 1982). The suspensions were incubated in septum-sealed vials and 1-ml headspace samples were removed with a gas-tight syringe and analyzed for ethane by gas chromatography under the following conditions:

Instrument: Hewlett-Packard Model 5710 gas chromatograph

Column: 2 mm x 2.0 m glass column packed with 80/100 mesh Porapak QS

Flow Rate: 30 ml/min N<sub>2</sub>

Temperature: 75°C Isothermal

Detector: Flame ionization

Integrator: Hewlett-Packard 3380A

Retention Time: 0.73 min (ethane)

Quantitation was achieved by the external standard method from a regression equation derived from standards over the range of 300 to 3000 ng ( $r^2 = 0.9972$ ).

Profiling of Hepatocyte Solutions for DNT Metabolites

Frozen hepatocyte suspensions were thawed and filtered through a 0.45- $\mu$  membrane filter. The aqueous filtrates were analyzed by high-performance liquid chromatography under the following conditions:

Instrument: Spectra-Physics 3500B Liquid Chromatograph

Column: Water's C<sub>18</sub>-NOVAPAK cartridge contained in a Water's Radial Compression Module

Mobile Phase: A-85% Phosphate buffer solution in 15% methanol  
(phosphate buffer prepared by dissolving 0.1 g of NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O and 0.375 g of Na<sub>2</sub>HO<sub>4</sub> in 1.0 liter of Milli-Q water)

B-15% Phosphate buffer solution in  
85% methanol

Gradient Program: 0% B (hold 2 min) —————> 50% B in 20 min

50% B (hold 10 min) —————> 90% B in 1 min

Flow Rate: 1.4 ml/min

Detector: UV @ 254 nm

Integrator: Hewlett-Packard 3380A

## VI. RESULTS

### Protein Synthesis.

All DNT analogs inhibited the accumulation of radiolabelled L-valine in hepatocyte proteins in a concentration-dependent manner. The data are summarized in Table 1. Inhibition with 2,5-, 2,3-, and 3,4-DNT was readily quantitated in the concentration range of 0.08 to 0.4 mM, whereas with 3,5-, 2,4-, and 2,6-DNT the response extended to concentrations of 1.2 mM and above for comparable degrees of inhibition.

The data from Table 1 are plotted in Figure 1 to allow estimation of IC<sub>50</sub> values, the concentration of the DNTs that inhibit the rate of protein synthesis by 50%. These values are taken from traces drawn through the data points that intersect the 50% line. The values, in the right-hand column of Table 1 are: 2,5-DNT, 0.16 mM; 1,4-DNT, 0.24 mM; 2,3-DNT, 0.25 mM; 2,4-DNT, 0.52 mM; and 2,6-DNT, 0.52 mM; 3,5-DNT, 0.53 mM.

### Lipid Peroxidation

Lipid peroxidation in DNT-treated flasks was assessed by quantitation of TBA reactants, the most commonly used and convenient technique for that purpose (Kappus, 1985). Since MDA, the lipid breakdown product that the TBA reaction primarily measures, can peak early during the incubation and in some cases can escape detection altogether because of metabolism in the mitochondria (Recknagel et al., 1982), TBA reactants were determined at both 30 and 240 min after DNT treatment. Cumene hydroperoxide was used as a positive control.

The results of the TBA reactants assay are summarized in Table 2. Cumene hydroperoxide produced a statistically significant increase ( $p < 0.01$  in the Student's t-test) in TBA reactants relative to control flasks at 30 min with, the difference increasing during an additional 210 min of incubation. The means of two separate experiments with the various DNT analogs showed no appreciable increase except possibly for 2,5-DNT at 240 min.

Measurement of ethane evolution using 2,5- and 3,5-DNT was attempted as an alternative indicator for lipid peroxidation. Ethane is produced in the process from the breakdown of linolenic acid (Wendel and Dumelin, 1981). In two experiments--cumene hydroperoxide produced 25.2 and 17.0 pmol of ethane per milliliter of headspace--well above the level of detection (5.0 pmol/ml) with the particular experimental protocol used. Only one of four flasks containing 2,5-DNT at 0.40 mM produced an increase (11.8 pmol/ml headspace) and none of four flasks monitored containing 3,5-DNT produced a detectable increase during 2 hr of incubation. (With this technique, the incubation time is shortened to avoid anoxia in the sealed flasks, because of the smaller headspace required to optimize ethane detection and measurement). Because reproducible results were not obtained and the results with 3,5-DNT were negative, attempts to measure lipid peroxidation in DNT-exposed hepatocyte suspensions were discontinued.

Table 1  
SUMMARY OF PROTEIN SYNTHESIS DATA<sup>a</sup>

(DNT Analog)	Concentration (mM)	% of Control Level	$IC_{50}^b$ (mM)
None	---	100	
2,4-DNT	0.25	75.5 (64.0, 86.9)	0.52
	0.45	55.5 ± 11.6 (4)	
	0.80	32.2 ± 19.1 (4)	
	1.2	26.3 ± 7.5 (4)	
2,6-DNT	0.25	70.7 (53.6, 87.8)	0.52
	0.45	59.1 ± 19.9 (4)	
	0.80	32.2 ± 7.0 (4)	
	1.2	19.1 ± 3.8 (4)	
3,5-DNT	0.25	77.8 (62.9, 92.8)	0.53
	0.45	61.7 (51.4, 72.1)	
	0.80	28.8 ± 21.5 (3)	
	1.2	23.5 (20.9, 26.1)	
	1.5	7.0 ± 4.7 (3)	
2,3-DNT	0.08	71.0 (69.8, 73.0)	0.25
	0.10	91.1 (83.6, 98.6)	
	0.20	59.2 ± 29.1 (4)	
	0.30	30.8 (29.4, 32.3)	
	0.40	3.0 (3.3, 2.7)	
3,4-DNT	0.10	99.4 (98.9, 100.1)	0.24
	0.20	45.5 ± 22.4 (4)	
	0.25	30.2 (24.0, 36.5)	
	0.30	13.8 ± 12.5 (4)	
2,5-DNT	0.10	72.3 ± 6.7 (4)	0.16
	0.20	30.4 ± 3.8 (4)	
	0.25	24.0 (25.4, 22.5)	
	0.30	17.2 ± 4.3 (4)	

<sup>a</sup>Means ± SD for a number of separate experiments given in parentheses for n = 3 or more. For n = 2, means plus data from individual experiments given in parentheses.

<sup>b</sup>Concentration for 50% inhibition of protein synthesis interpolated from plots of % protein synthesis vs. DNT concentration in the medium (Figure 1).

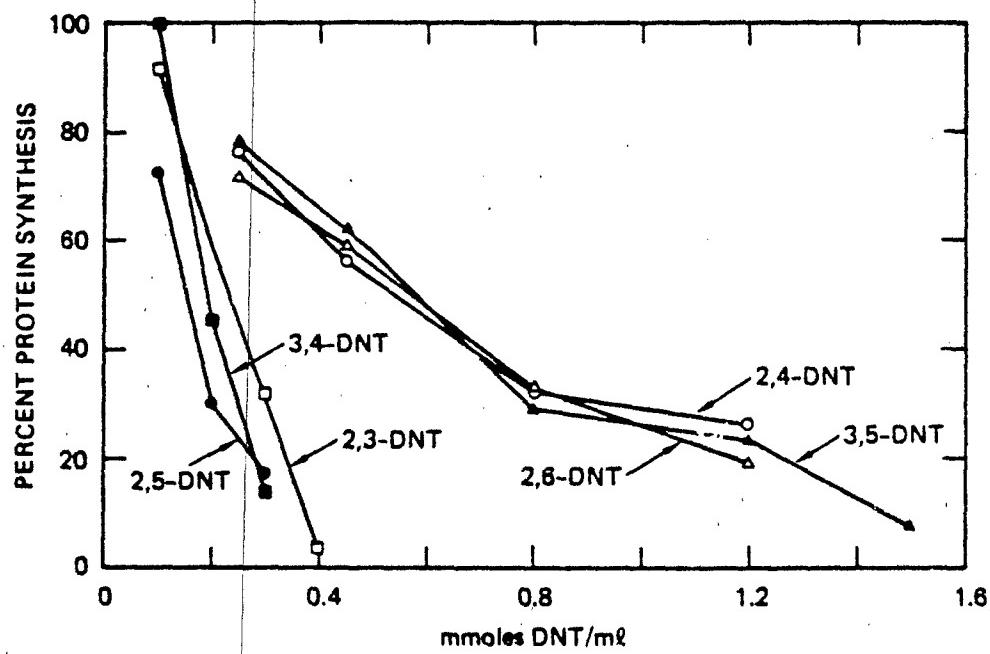


FIGURE 1 EFFECT OF DNT ISOMERS ON PROTEIN SYNTHESIS

Table 2  
SUMMARY OF TBA REACTANTS ASSAY DATA<sup>a</sup>

<u>Compound</u>	<u>Concentration (mM)</u>	<u>MDA (mmol/10<sup>6</sup> cells)</u>	
		<u>DNT Flasks</u>	<u>Corresponding Control Flasks</u>
<u>At 30 min</u>			
2,4-DNT	1.2	1.44 (1.78, 0.96)	1.46 (1.82, 1.09)
	2.0	1.86 (1.65, 2.06)	
2,6-DNT	1.2	0.52 (0.84, 0.18)	0.96 (1.49, 0.42)
	2.0	0.77 (1.11, 0.42)	
3,5-DNT	1.2	1.08 (1.68, 0.48)	1.46 (1.82, 1.09)
	2.0	1.35 (1.66, 1.03)	
2,3-DNT	0.30	1.36 (2.48, 0.23)	1.14 (1.82, 0.42)
	0.40	0.92 (1.66, 0.18)	
3,4-DNT	0.30	1.06 (1.62, 0.49)	0.96 (1.49, 0.42)
	0.40	1.19 (1.65, 0.73)	
2,5-DNT	0.30	1.06 (0.32, 1.81)	1.29 (1.09, 1.49)
	0.40	1.49 (1.14, 1.84)	
Cumene hydroperoxide	1.2	3.83 ± 0.92 (4)	1.21 ± 0.60 (4)
<u>At 240 min</u>			
2,4-DNT	1.2	0.96	0.75
	2.0	1.20	
2,6-DNT	1.2	0.74 (0.97, 0.50)	0.90 (1.12, 0.68)
	2.0	1.24 (1.84, 0.65)	
3,5-DNT	1.2	0.51	0.75
	2.0	0.92	
2,3-DNT	0.3	0.64	0.68
	0.4	0.38	
3,4-DNT	0.3	1.27 (1.81, 0.73)	0.90 (1.12, 0.68)
	0.4	1.06 (1.53, 0.63)	
2,5-DNT	0.3	1.30 (0.87, 1.72)	0.94 (0.75, 1.12)
	0.4	1.90 (1.48, 2.31)	
Cumene hydroperoxide	1.2	4.97 ± 1.18 (3)	0.85 ± 0.24)

<sup>a</sup>Means ± SD with number of separate experiments in parentheses for n = 3 or more. For n<3, means plus data for individual experiments in parentheses.

### LDH Release

All DNTs produced an increased loss of intracellular LDH to the medium over control cell loss during the 4-hr incubation at one or more test concentrations in initial experiments. Early in the course of conducting these assays, however, reports found in the literature suggested modifications in the experimental conditions (from those originally proposed by us) for more valid results. Specifically, it was recommended that an air:CO<sub>2</sub> (95:5) rather than a carbogen, or O<sub>2</sub>:CO<sub>2</sub> (95:5), atmosphere be used in cytotoxicity studies with isolated hepatocytes (Bridges et al., 1983). In one experiment under the incubation conditions used by us with 2,4-, 2,3- and 3,5-DNT, the LDH release for 3,5-DNT under a carbogen atmosphere was greater than under an air:CO<sub>2</sub> atmosphere; the difference was enough to suggest a possible effect of O<sub>2</sub> content on the cytotoxicity of one or more DNTs. Furthermore, it was noted that subdued lighting was used in some studies with DNTs, presumably because of concern that the potential photoreactivity of the chemicals might impact on results during the incubation although data to this effect were not presented (Bond and Rickert 1981). An experiment with 2,4-, 2,6-, and 2,5-DNT in flasks exposed to normal laboratory lighting and covered to prevent contact with light seemed to confirm a potential light/dark effect on net LDH release. However, the light/dark effect noted above was not consistently reproducible, and other potential sources of variability were identified, e.g., different design features in the water baths used as well as (possibly most important) inherent and considerable variability at times in LDH release as an endpoint indicator of toxicity in replicate flasks. It was therefore decided to pool all experimental data on LDH release in experiments under an air:CO<sub>2</sub> atmosphere.

The data from these experiments are presented in Table 3. The DNTs produced increases in percent LDH release from the hepatocytes in a concentration-dependent manner. (Not all data obtained in these experiments are presented in the table to allow focusing on the concentration range in which a significant increase in LDH release is observed.) Concentrations of 2,4-, 2,6- and 3,5-DNT are solubility-limited in the medium, with fine precipitates being evident in the range of 1.5 to 2.0 mM. The data in Table 3 indicate that the o- and p-substituted DNTs enhance LDH release at 5- to 10-fold lower concentrations than do the m-substituted analogs. An approximate potency ranking appears to be 3,4- > 2,5- > 2,3- > 2,6- > 3,5- > 2,4-DNT.

The DNTs may be ranked for cytotoxic potency by plotting the data in Table 3 and interpolating EC<sub>50</sub> values from visual best-fit traces through the data points (Figure 2). The EC<sub>50</sub> value corresponds in our experiments to an approximate doubling of cell death in the DNT-treated flasks in comparison with solvent-only controls. For the 6 DNTs, the values are: for 3,4-DNT, 0.30 mM; for 2,5-DNT, 0.41 mM; for 2,3-DNT, 0.48 mM; for 2,6-DNT, 1.02 mM; for 3,5-DNT, 1.76 mM; and for 2,4-DNT, 1.79 mM.

### Chemical Metabolism

Each solution of hepatocytes was filtered and the aqueous filtrate was analyzed directly by HPLC using gradient elution and UV detection as described in the Methods section. The metabolites were identified by co-elution of authentic standards when available and some peaks were collected for identification by probe-mass spectrometry evaluation. The available standards were

related to 2,4-dinitrotoluene and consisted of 2-amino-4-nitrotoluene, 4-amino-2-nitrotoluene, 4-N-acetyl amino-2-nitrotoluene, and 2-amino-4-nitrobenzyl alcohol. An HPLC profile of the standards appears in Figure 3. Using these standards as a guide, we estimated the major metabolites produced by hepatocytes.

Table 3  
SUMMARY OF NET PERCENT LDH RELEASE<sup>a</sup>

<u>Compound</u>	<u>Concentration (mM)</u>	<u>Net % LDH Release</u>
2,4-DNT	1.2	4.1 ± 2.4 (5)
	1.5	6.5 ± 5.2 (5)
	2.0	34.2 ± 26.8 (8)
2,6-DNT	0.80	13.0 ± 9.2 (6)
	1.2	24.4 ± 19.2 (6)
	1.5	38.2 ± 22.5 (8)
	2.0	43.7 ± 31.8 (4)
3,5-DNT	1.2	2.8 (4.9, 0.8)
	1.5	6.8 ± 6.5 (6)
	1.8	21.9 ± 12.8 (3)
	2.0	25.3 ± 19.4 (7)
2,3-DNT	0.20	4.4 (7.6, 1.2)
	0.30	0.35 (0.0, 0.70)
	0.35	13.8 (23.0, 4.7)
	0.40	10.9 ± 9.7 (5)
	0.50	39.8 ± 39.9 (5)
	0.60	21.1 ± 14.2 (3)
	1.0	78.9 ± (75.2, 82.6)
	1.5	85.9 (87.8, 84.0)
	2.0	98.4 (100, 96.6)
2,5-DNT	0.25	3.5 ± 21 (3)
	0.30	16.2 ± 6.4 (3)
	0.35	7.0 ± 11.0 (3)
	0.40	19.2 ± 18.4 (7)
	0.50	55.0 ± 40.4 (7)
	0.60	30.1 (44.6, 15.6)
	1.0	78.9
	1.5	85.9
	2.0	98.8
3,4-DNT	0.25	8.1 ± 5.9 (3)
	0.30	29.2 ± 29.8 (6)
	0.35	35.0 ± 22.4 (3)
	0.40	44.8 ± 30.5 (4)
	0.50	50.6 (84.0, 17.1)

<sup>a</sup>Means ± SD with number of separate experiments in parenthesis for n = 3 or more. For n = 2, means plus data for individual experiments in parentheses for r < 3.

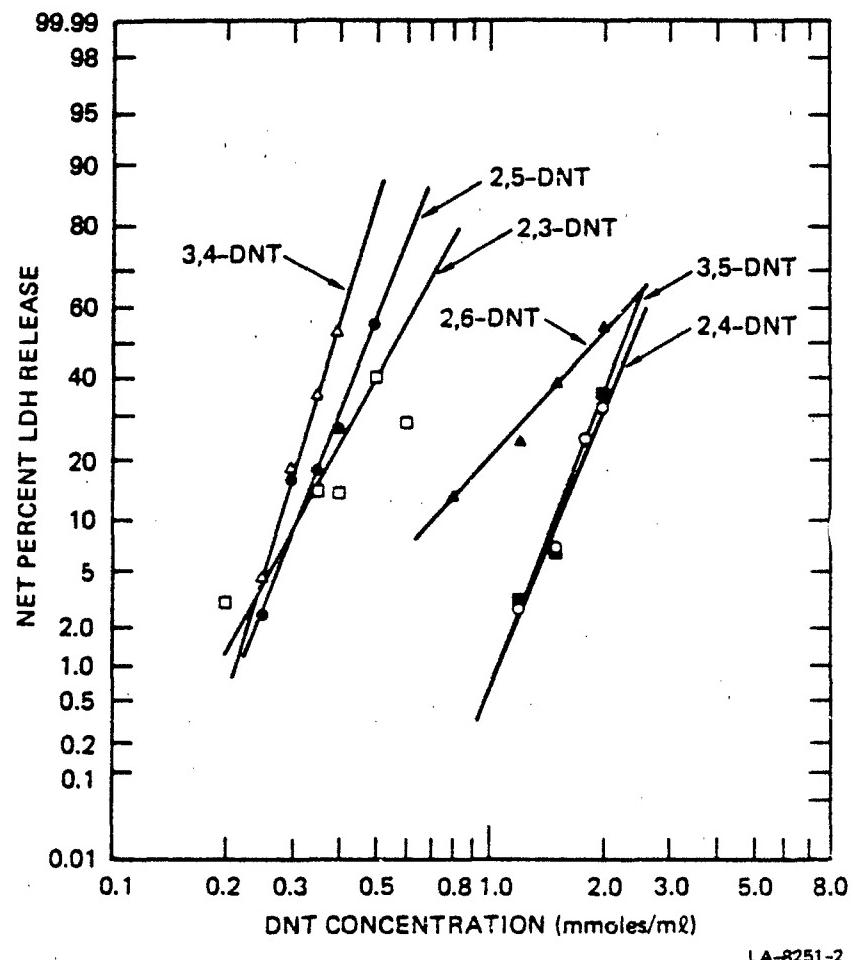


FIGURE 2 EFFECT OF DNT CONCENTRATION ON PERCENT LDH RELEASE

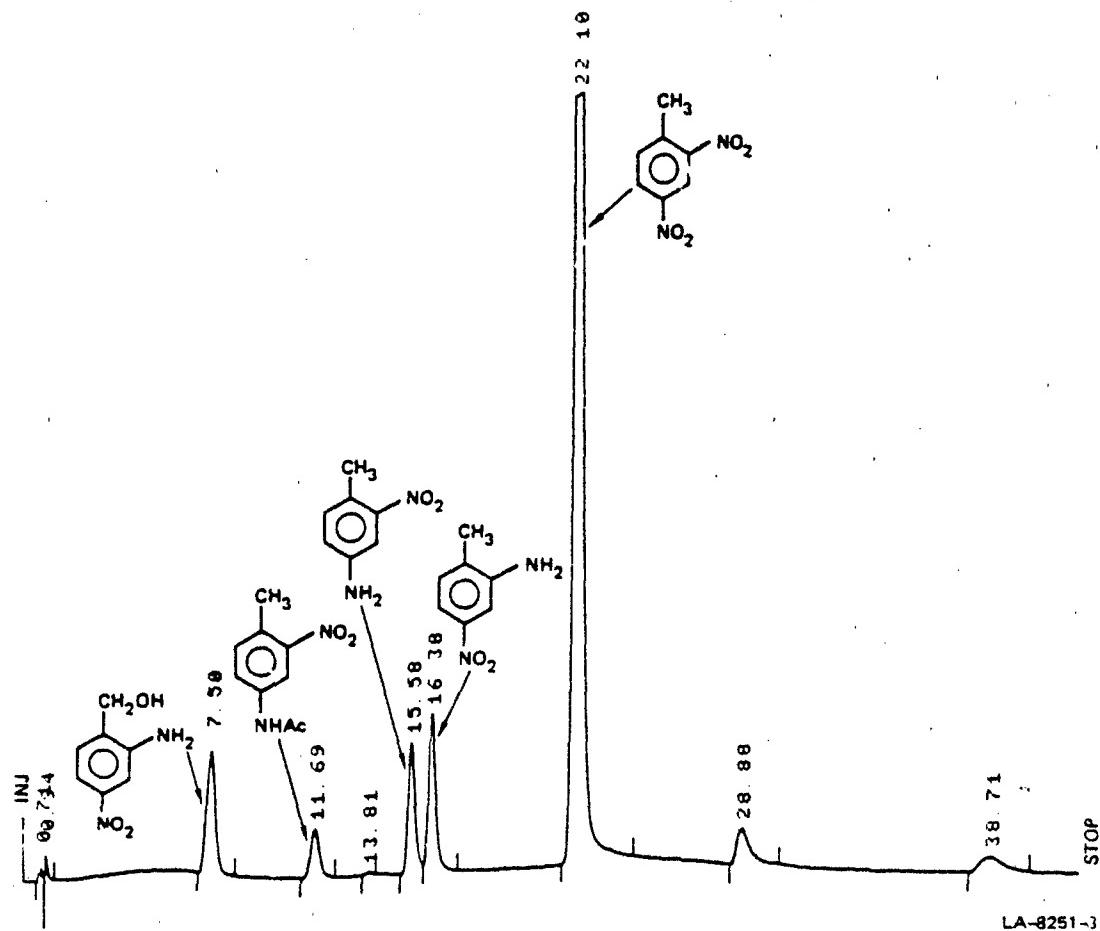


FIGURE 3 HPLC PROFILE OF 2,4-DINITROTOLUENE STANDARDS

#### 2,4-Dinitrotoluene (2,4-DNT)

The HPLC profile of 2,4-DNT solution after 4 hr of incubation with hepatocytes is shown in Figure 4. The compounds that were identified by comparative chromatography and confirmed by probe mass spectroscopy were 4-amino-2-nitrotoluene, 2-amino-4-nitrotoluene, and 4-acetylamino-2-nitrotoluene. The structures of these compounds are also shown in Figure 4. Purging of the column with 90% of Solvent B eluted components supposedly less polar than the parent compound. These components could not be identified but could potentially be related to condensed azo and azoxy derivatives of 2,4-DNT. The treatment of the hepatocyte suspension with a  $\beta$ -glucuronidase enzyme preparation did not produce 2,4-dinitrobenzyl alcohol. These data suggest that very little, if any, oxidative transformations have occurred with 2,4-DNT.

#### 2,6-Dinitrotoluene (2,6-DNT)

The HPLC profile of the 2,6-DNT hepatocyte solution after 4 hrs of incubation with hepatocytes is shown in Figure 5. One metabolite was identified as 2-amino-6-nitrotoluene by chromatographic comparison with an authentic standard (Aldrich Chemical Co.). The component eluting at 7.99 min was collected and evaluated by probe mass spectrometry. The negative chemical ionization (NCI) spectrum gave a molecular weight of 198 (Figure 6), and following silylation, a molecular weight of 270 (Figure 7). These data are consistent with the compound being 2,6-dinitrobenzyl alcohol. Thus, in 2,6-DNT metabolism by hepatocytes, both oxidative and reductive transformations appear to be occurring.

#### 3,5-Dinitrotoluene (3,5-DNT)

The HPLC profile of the 3,5-DNT solution after 4 hr of incubation with hepatocytes appears in Figure 8. A major metabolite was identified to be 3-amino-5-nitrotoluene based on chromatographic comparison with an authentic standard (18.84 min). A minor component eluting at 13.54 min was tentatively identified as 3-acetylamino-5-nitrotoluene based on its elution time as compared with that of 4-acetylamino-2-nitrotoluene. No oxidative transformations are apparent from the HPLC profile.

#### 2,5-Dinitrotoluene (2,5-DNT)

The HPLC profile of the 2,5-DNT solution after 4 hr of incubation with hepatocytes appears in Figure 9. The parent compound (eluting at 22.38 min) was transformed quite rapidly and produced 3-amino-6-nitrotoluene and 2-amino-5-nitrotoluene, which were identified by mass spectroscopy of collected fractions. These isomers are readily distinguished by mass spectrometry since nitro groups oriented ortho to a methyl group show a characteristic mass loss of 17 (O-H) that was observed with 3-amino-6-nitrotoluene and not with 2-amino-5-nitrotoluene. The component eluting at 8.59 min suggests a polar intermediate (hydroxylamine?), but its chromatography does not suggest the benzyl alcohol. It appears that oxidative transformation is small compared with reductive transformation in 2,5-DNT.

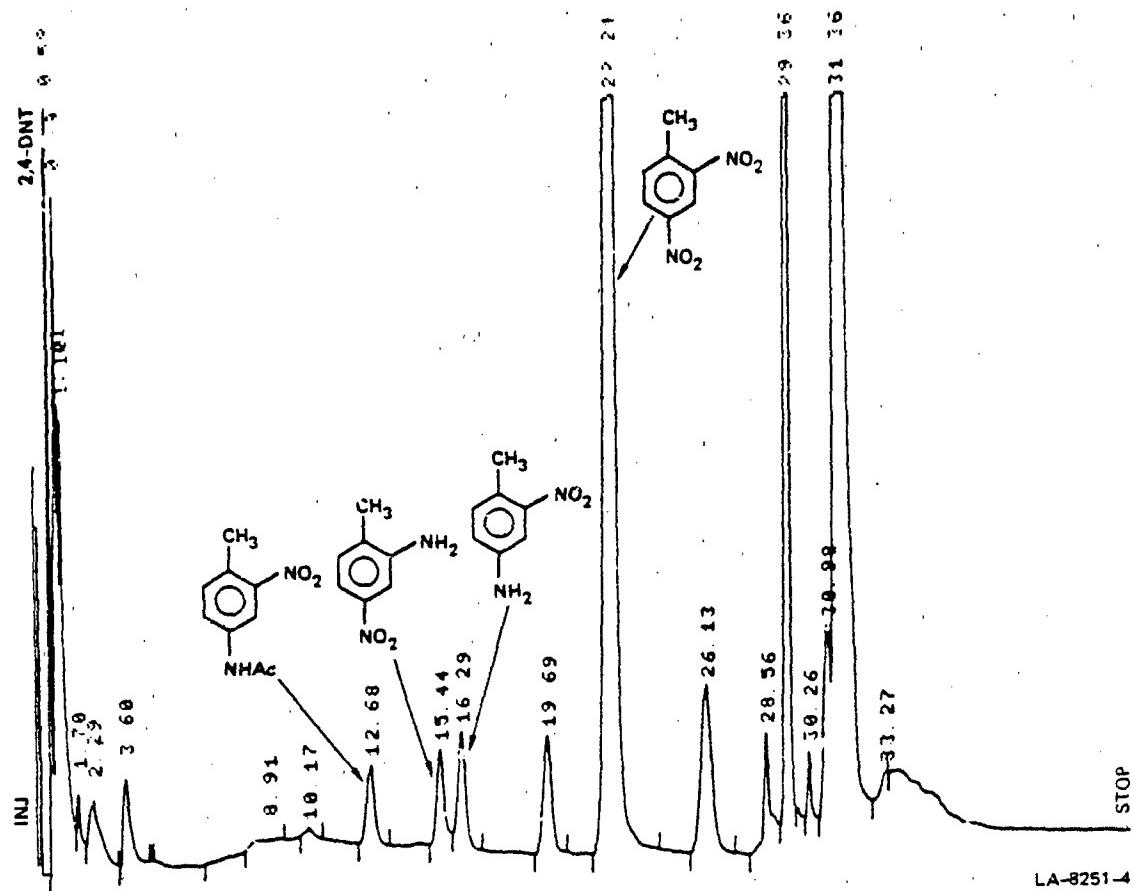


FIGURE 4 HPLC PROFILE OF 2,4-DNT SOLUTION IN THE PRESENCE OF HEPATOCYTE SOLUTION

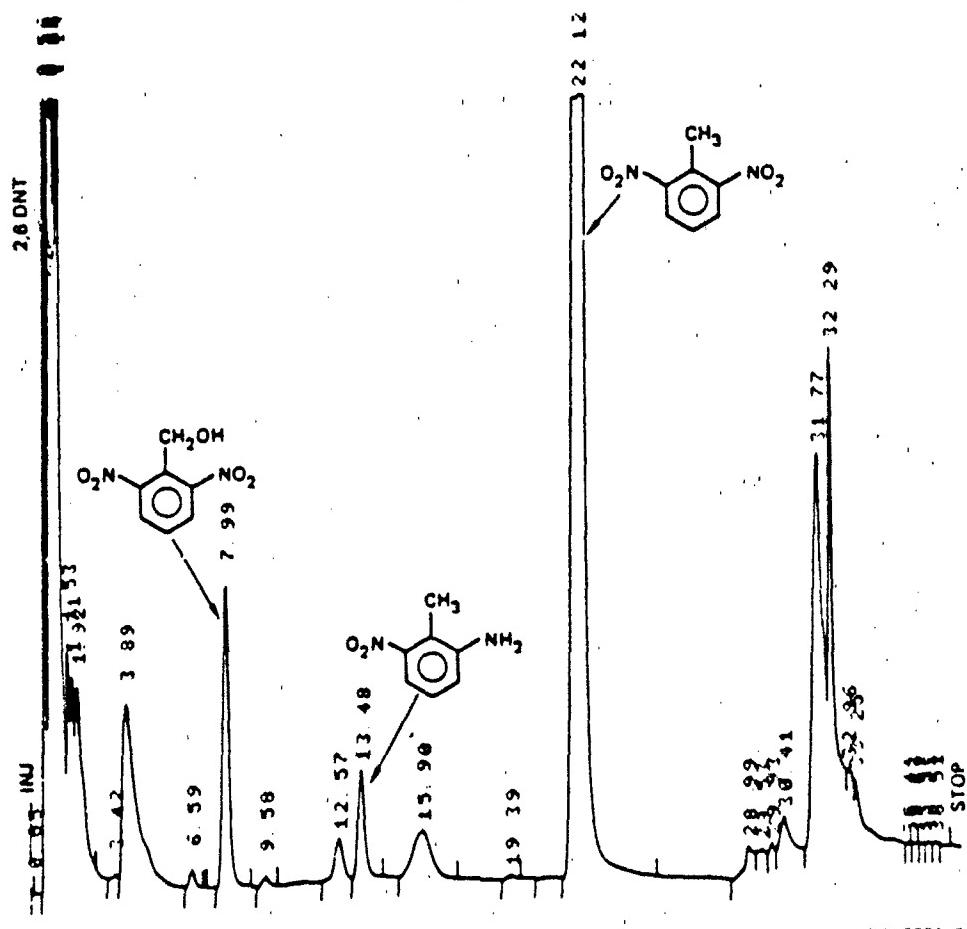


FIGURE 5 HPLC PROFILE OF 2,6-DNT-HEPATOCYTE SOLUTION

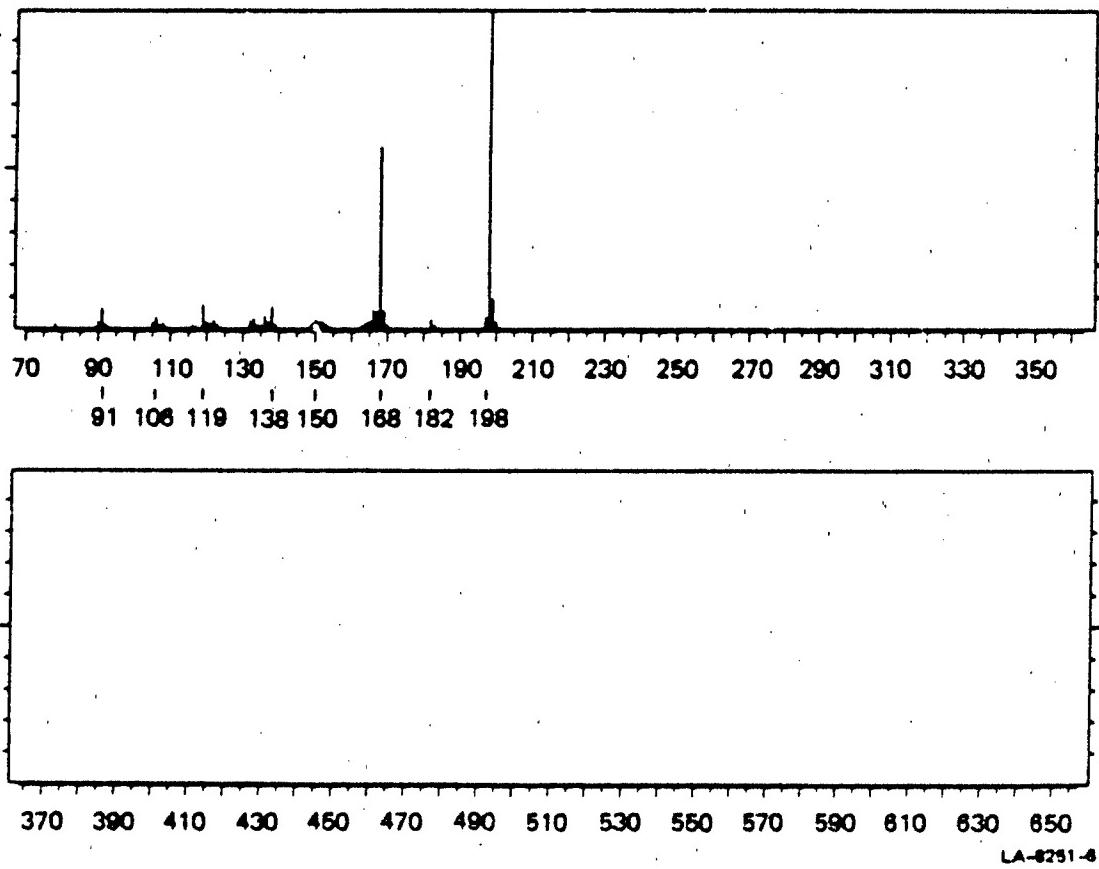


FIGURE 6 NCI MASS SPECTRUM OF 2,6-DINITROBENZYL ALCOHOL

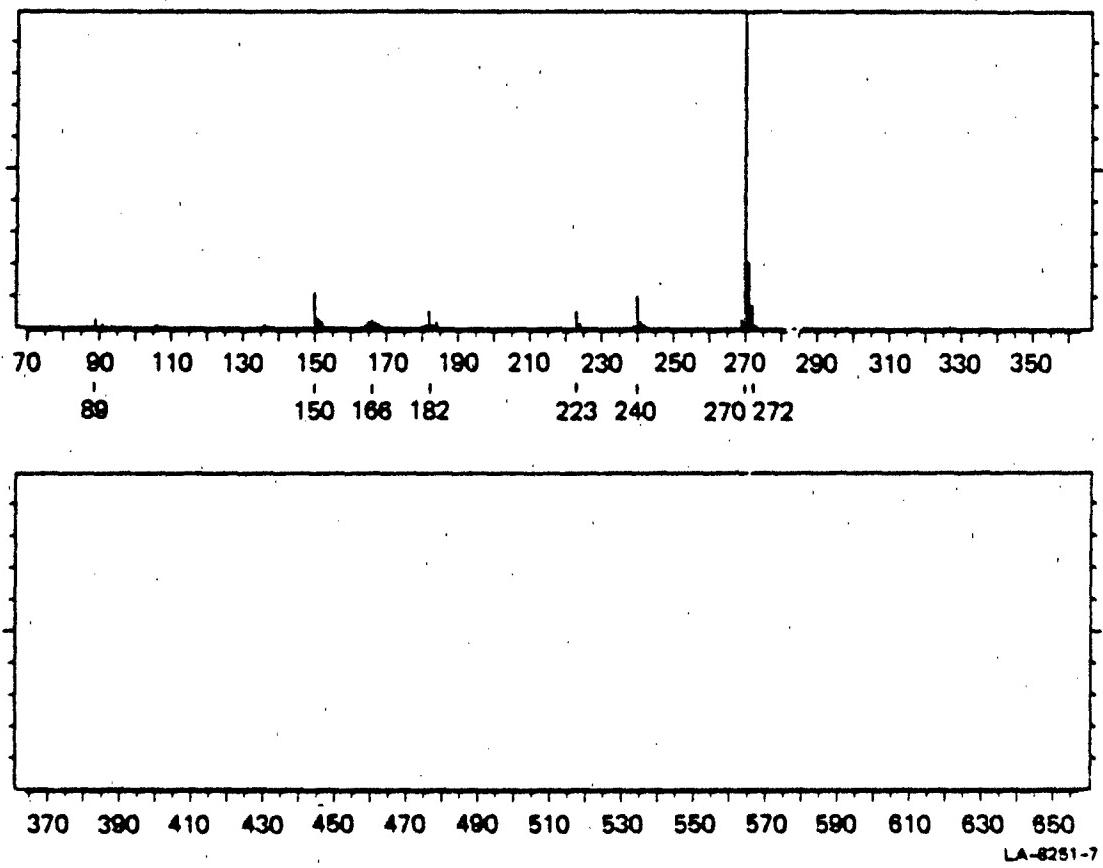


FIGURE 7 NCI MASS SPECTRUM OF SILYLATED 2,6-DINITROBENZYL ALCOHOL

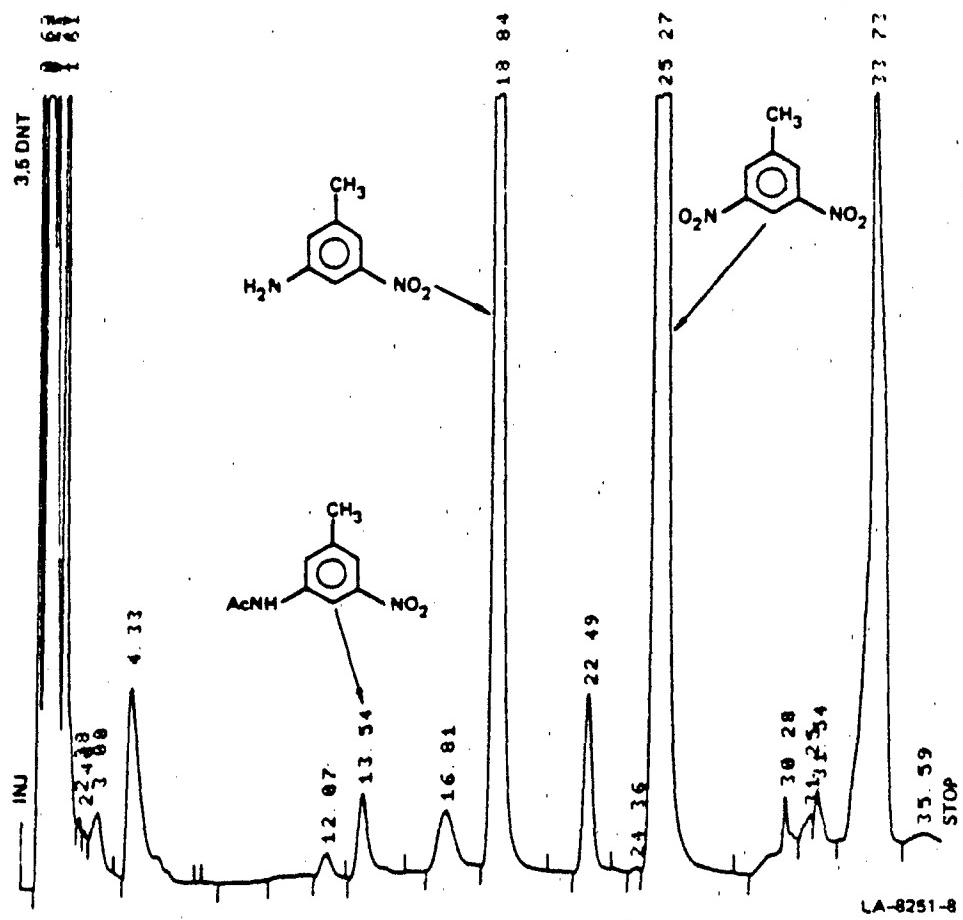


FIGURE 8 HPLC PROFILE OF 3,5-DNT-HEPATOCYTE SOLUTION

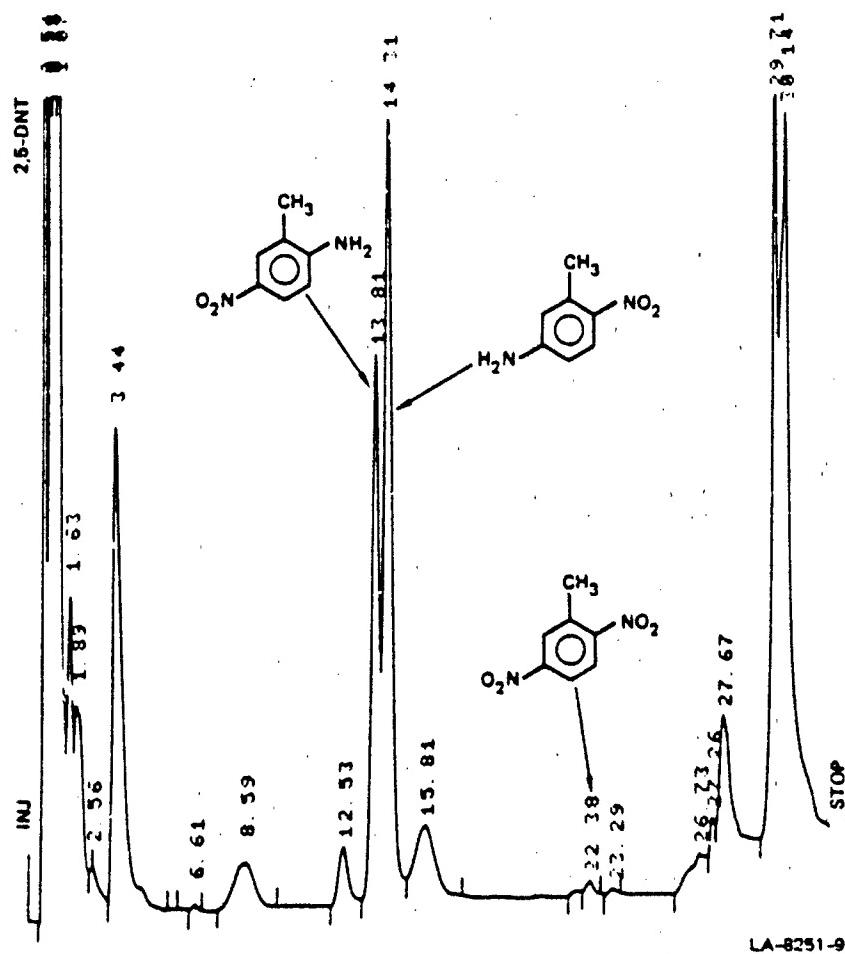
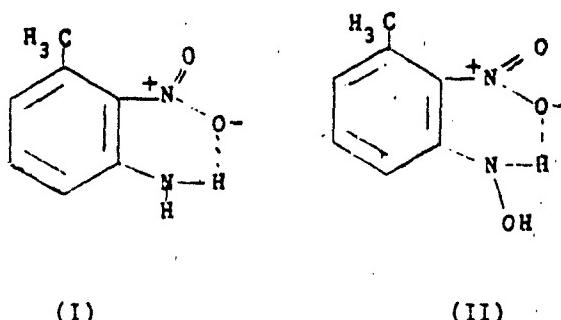


FIGURE 9 HPLC PROFILE OF THE 2,5-DNT-HEPATOCYTE SOLUTION

### 2,3-Dinitrotoluene (2,3-DNT)

The HPLC profile of the 2,3-DNT solution after 4 hr of incubation with hepatocytes appears in Figure 10. A major metabolite was identified as 3-amino-2-nitrotoluene by chromatographic comparison with an authentic standard (Aldrich Chemical Co.). As observed in the case of 2,5-DNT, a broad-polar component, which was not identified, eluted at 6.46 min. The long elution time of the amino metabolite suggests that there may be some intramolecular hydrogen bonding between the neighboring amino and nitro group. This may retard further reduction of the nitro group or acetylation of the amino group and add stability to nitro-amino or nitro-hydroxylamino functionalities (I and II).



(I)

(II)

### 3,4-Dinitrotoluene (3,4-DNT)

The HPLC profile of the 3,4-DNT solution after 4 hr of incubation with hepatocytes appears in Figure 11. One metabolite was identified as 4-amino-3-nitrotoluene by chromatographic comparison with an authentic standard. This peak (21.99 min) is poorly shaped, which suggests that the 3-amino isomer may be co-eluting with the 4-amino-isomer. The long retention time again suggests intramolecular associations, as observed with 2,3-DNT. A major and broad-eluting metabolite appears at 7.33 min. Although this is the area of the profile where benzyl alcohols elute, the chromatography, as observed by us for 2,6-dinitrobenzyl alcohol and by Bond and Rickert (1981) for 2,4-dinitrobenzyl alcohol, does not suggest the alcohol but, rather, the hydroxylamine. No spectral data could be obtained for this metabolite.

#### Fecal Enzymes.

To determine whether reductive metabolites produced in the gut can contribute to the hepatotoxicity of DNT analogs, an attempt was made to generate these *in vitro* by incubation of the parent compound with gut microflora and transfer of the mixture to the hepatocyte medium for evidence of increased LDH release under normal incubation conditions. Results of the evaluation of 2,5-, 2,6-, and 2,4-DNT this method are summarized in Table 4. At the maximum concentration of each in the medium that could be used, based on the solubility of the compound and the amount of fecal enzyme isolated from the cecum of

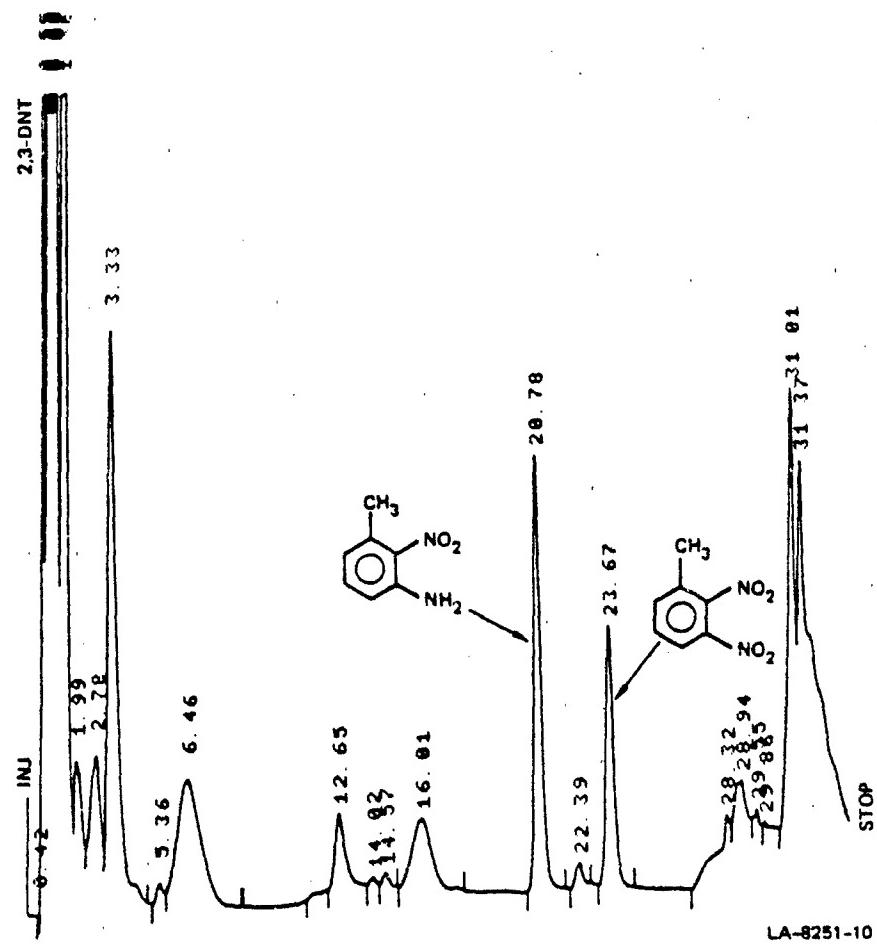


FIGURE 10 HPLC PROFILE OF THE 2,3-DNT-HEPATOCYTE SOLUTION

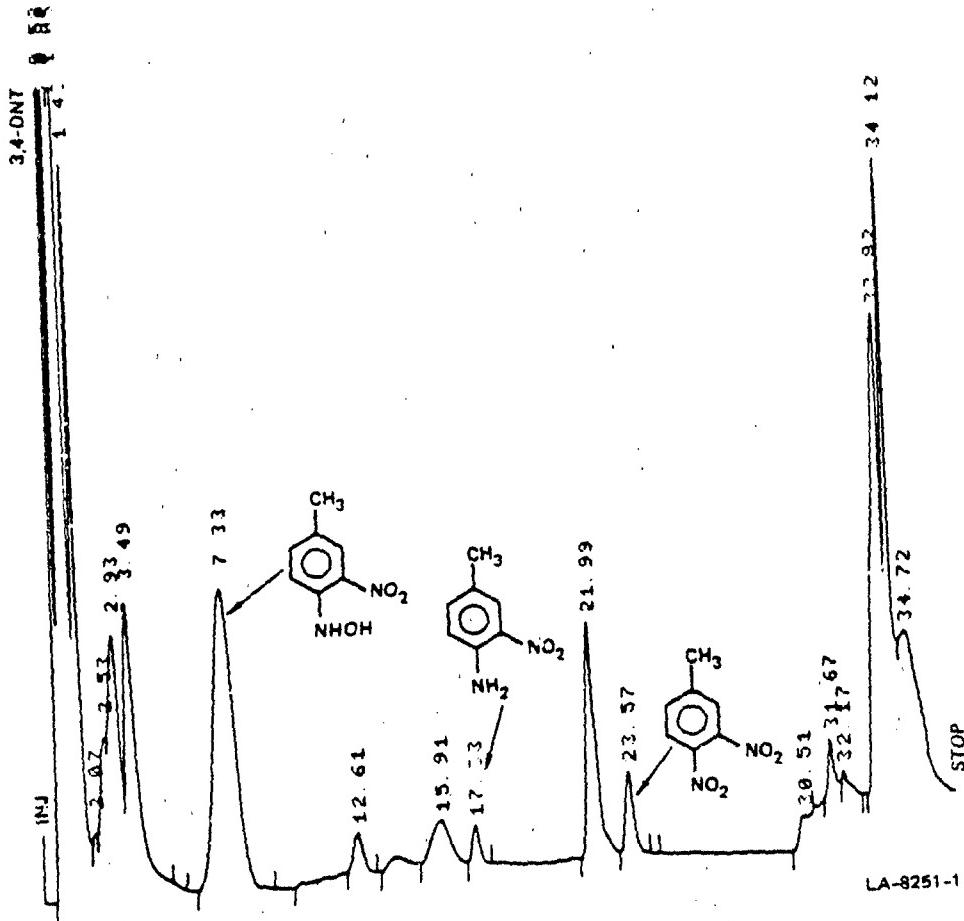


FIGURE 11 HPLC PROFILE OF THE 3,4-DNT-HEPATOCYTE SOLUTION

two rats, the data indicate no enhanced LDH release with either 2,5- or 2,4-DNT and a small but insignificant increase with 2,6-DNT. The total amine content of the fecal specimens introduced into the incubation medium was no more than 2 to 3%, however. With 2,6- and 2,4-DNT unidentified peaks at longer retention times than the parent compound constituted 20 and 10% of the total, respectively.

Table 4  
SUMMARY OF GUT MICROFLORA STUDIES

<u>Compound</u>	Concentration (mM)	% LDH Release	
		+gm*	-gm
2,5-DNT	0.5	2.1 ± 1.9 50.3 ± 35.6	42.4 ± 40.9 70.5 ± 0
2,6-DNT	0.75	1.5 ± 1.3 8.3 ± 9.4	0 ± 0 1.4 ± 2.5
<u>2,4-DNT</u>	1.0	5.4 ± 4.0 2.7 ± 2.6	10.1 ± 8.2 7.9 ± 2.3

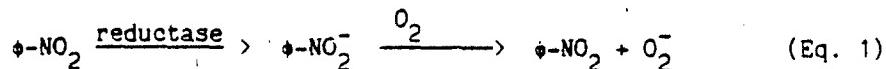
\* gm = gut microflora

## VII. DISCUSSION

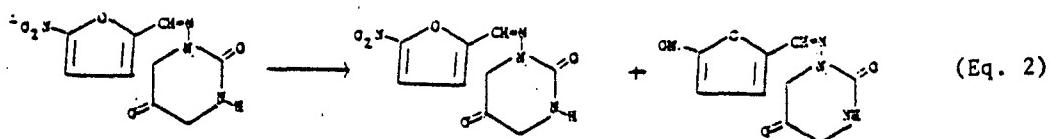
The cytotoxicity of DNT isomers to rat hepatocytes was evaluated by three biological parameters: LDH release, protein synthesis, and lipid peroxidation. The LDH data, as shown in Figure 2, distribute the isomers into two distinct groups; one group containing 2,3-, 3,4-, and 2,5-DNT is active at appreciably lower concentrations than the other group, which contains 2,6-, 2,4-, and 3,5-DNT. In the first group the nitro groups are oriented ortho-para to each other, whereas in the second group the nitro groups are oriented meta to each other. Thus, cytotoxicity appears to be related to the nitro group orientation. It is interesting to note that the slopes of the lines in Figure 2 are nearly parallel for all isomers except 2,6-DNT. This suggests that the principal metabolites responsible for cytotoxicity may be different in the case of 2,6-DNT compared with the other isomers. One of the distinguishing features of the metabolic profiles of the DNT isomers was that the only isomer which appeared to exhibit predominantly oxidative transformation was 2,6-DNT.

The effect of the DNT isomers on protein synthesis paralleled the same effects observed in LDH release. Isomers in the ortho-para-oriented nitro group were more potent inhibitors of protein synthesis than the meta-oriented isomers. Also the slopes of the dose-response curves paralleled each other within each group but deviated significantly between groups (Figure 1). The fact that these changes in protein synthesis rates were observed at lower DNT concentrations than for LDH release indicates that protein synthesis is a more sensitive indicator of toxicity, as found to be true for other chemicals (Bridges et al., 1983; Goethals et al., 1984).

The effect of the DNT isomers on lipid peroxidation in hepatocytes was not consistently observed under any conditions using either TBA reactants or ethane production as a measure of an effect. Thus, it was difficult to associate free radical activity with cytotoxicity. This was somewhat surprising because all of the isomers showed some degree of reductive metabolism. Since the reduction of a nitro group to an amino group involves the transfer of six electrons, three free-radical intermediates are expected to be produced. Presumably the reaction (Eq. 1)



can occur (Holtzman et al., 1981), thereby generating superoxide radicals that can react with phospholipids in plasma membranes to produce breakdown products of peroxidation. However, radicals may be too short-lived in the oxygenated test system through the action of superoxide dismutase and peroxidases in the hepatocytes that protect the cells from injurious effects of the free radicals. Alternatively, this pathway may be minor compared with disproportionation of the DNT nitro free radical to the nitro and nitroso derivatives (Eq. 2), as occurs with nitrofuranoin (Holtzman et al., 1981):



If reduction of a nitro group is required to induce cytotoxicity, then the reduction potential of each isomer may be correlated with cytotoxicity. The reduction potentials of the DNT isomers have been studied by Tallec (1968) and Pearson (1948). Tallec studied all of the DNT isomers but only at low pH (pH-1.0). Pearson studied four of the DNT isomers as a function of pH and the combined results of the two authors appear in Table 5.

Table 5  
MEASURED 4-ELECTRON REDUCTION POTENTIALS OF DNT ISOMERS

Compound	Reduction Potential ( $E_{1/2}$ , volts)		
	pH 1.0 <sup>a</sup>	pH. 5.7 <sup>b</sup>	pH 7.4 <sup>b</sup>
2,6-DNT	-.130	-.410	-.460
3,5-DNT	-.070	---	---
2,4-DNT	-.090	-.350	-.400
2,5-DNT	-.020	---	---
2,3-DNT	-.090	-.340	-.400
3,4-DNT	-.070	-.310	-.360

<sup>a</sup>Tallec, 1968

<sup>b</sup>Pearson, 1948

These data suggest that 2,6-DNT is the most difficult to reduce and that 2,5-DNT is the easiest to reduce if the data are extrapolated to physiologic pH (7.4). A difficulty in correlating cytotoxicity with reduction potential is the similar reduction potentials reported for 2,3-DNT and 2,4-DNT and possibly for 3,4-DNT and 3,5-DNT, whereas each set of isomers displayed wide variations in cytotoxicity (of course, these are 4-electron reductions and only a 1-electron reduction may be necessary for toxicity). It is possible that enzymatic reductions proceed with different facility than do polarographic reductions because of stereospecific requirements at the enzyme active sites; and this would indicate a correlation of cytotoxicity with reduction potential. Tallec (1968) reports that the 5-nitro group in 2,5-DNT and the 4-nitro group in 2,4-DNT are reduced before the 2-nitro group. In our studies with 2,5-DNT and 2,4-DNT, reduction was nearly equivalent at each nitro group. (The only exception was 2,3-DNT, where the 3-position was the preferred

reduction site, in agreement with Tallec's report).

We alternatively attempted to correlate the cytotoxicity with electronic properties of the molecules using, first, the orbital energy of the lowest unoccupied molecular orbital (LUMO) as reported by Loew et al. (1985). The LUMO energies predicted by the MNDO molecular orbital method (Dewar and Thiel, 1977) appear in Table 6.

Table 6  
PREDICTED LUMO ENERGIES FOR DNT ISOMERS

<u>Compound</u>	<u>LUMO(electron volts)</u>
2,6-DNT	-1.786
2,4-DNT	-2.001
3,5-DNT	-2.039
2,3-DNT	-1.843
3,4-DNT	-1.973
2,5-DNT	-2.231

Again, there was no apparent trend in orbital energies of the ortho-para-oriented nitro groups compared with the meta-oriented DNT isomers that would permit development of theoretical model to predict hepatotoxic effects.

A relationship can be developed, however, if one compares the hepatotoxic effect to the electronic charges on the atoms of each isomer. The atomic charges on the ring and methyl carbon atoms relative to benzene were computed using the semiempirical quantum mechanical method, MNDO, for each DNT isomer. The results are shown in Table 7.

Table 7  
CALCULATED CARBON ATOM CHARGES FOR DNT ISOMERS RELATIVE TO BENZENE

DNT Isomer	C <sub>1</sub>	C <sub>2</sub>	C <sub>3</sub>	C <sub>4</sub>	C <sub>5</sub>	C <sub>6</sub>	C-Methyl
2,3-DNT	0.011	0.065	0.042	0.012	-0.025	-0.013	-0.018
3,4-DNT	-0.038	0.023	0.050	0.051	0.005	-0.002	-0.011
2,5-DNT	0.002	0.044	-0.006	0.017	0.009	0.026	-0.018
2,6-DNT	0.100	-0.014	0.050	-0.068	0.050	-0.014	-0.033
2,4-DNT	0.064	-0.018	0.089	-0.036	0.062	-0.068	-0.026
3,5-DNT	-0.098	0.081	-0.039	0.090	-0.039	0.081	-0.003

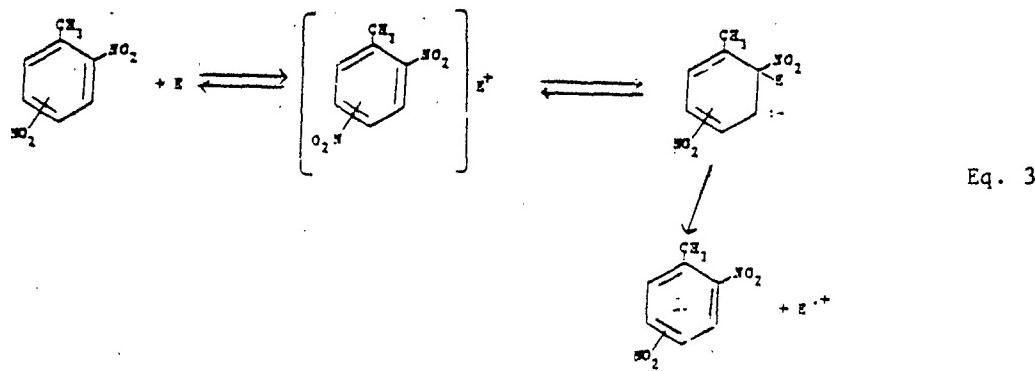
The charges on the carbon atoms bearing nitro groups are retabulated in Table 8.

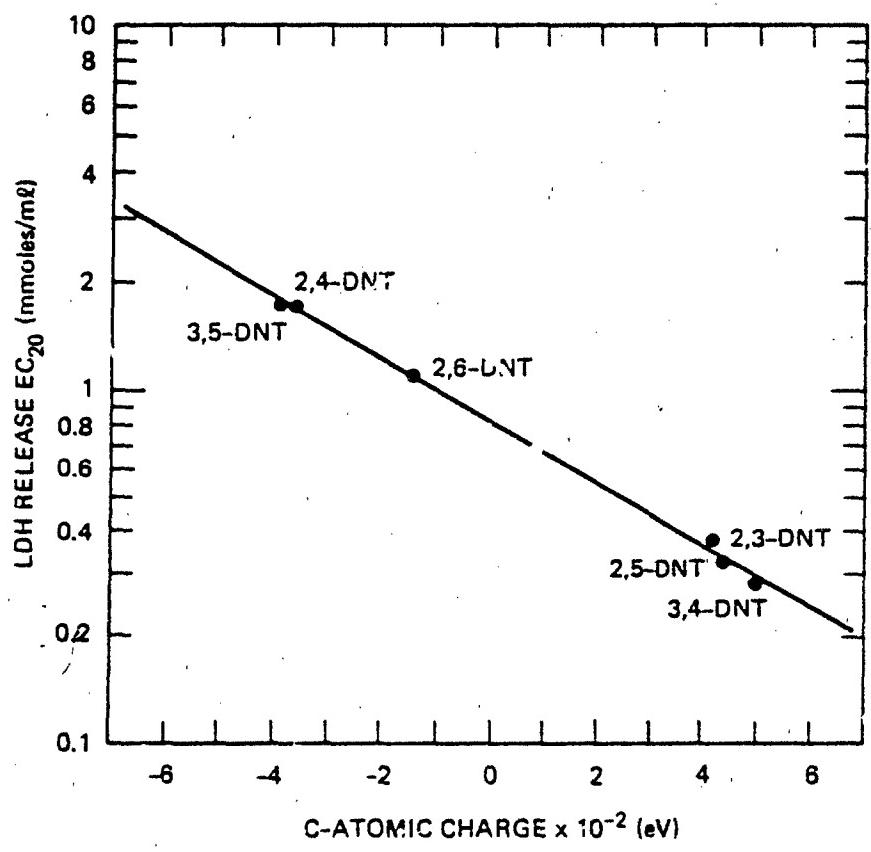
Table 8  
CARBON ATOM CHARGES FOR NITRO GROUPS BEARING CARBON ATOMS

DNT Isomer	I-NO <sub>2</sub>	II-NO <sub>2</sub>
2,3-DNT	0.065 (2)	0.042 (3)
3,4-DNT	0.050 (3)	0.051 (4)
2,5-DNT	0.044 (2)	0.009 (5)
2,6-DNT	-0.014 (2)	-0.014 (6)
2,4-DNT	-0.018 (2)	-0.036 (4)
3,5-DNT	-0.039 (3)	-0.039 (5)

The data in Table 8 (selected from Table 7) show that the carbon atoms bearing the nitro groups have positive atomic charges for the ortho-para-oriented isomers and negative atomic charges for the meta-oriented isomers. The data are plotted against EC<sub>50</sub> values for LDH release in Figure 12 and against protein synthesis inhibition in Figure 13.

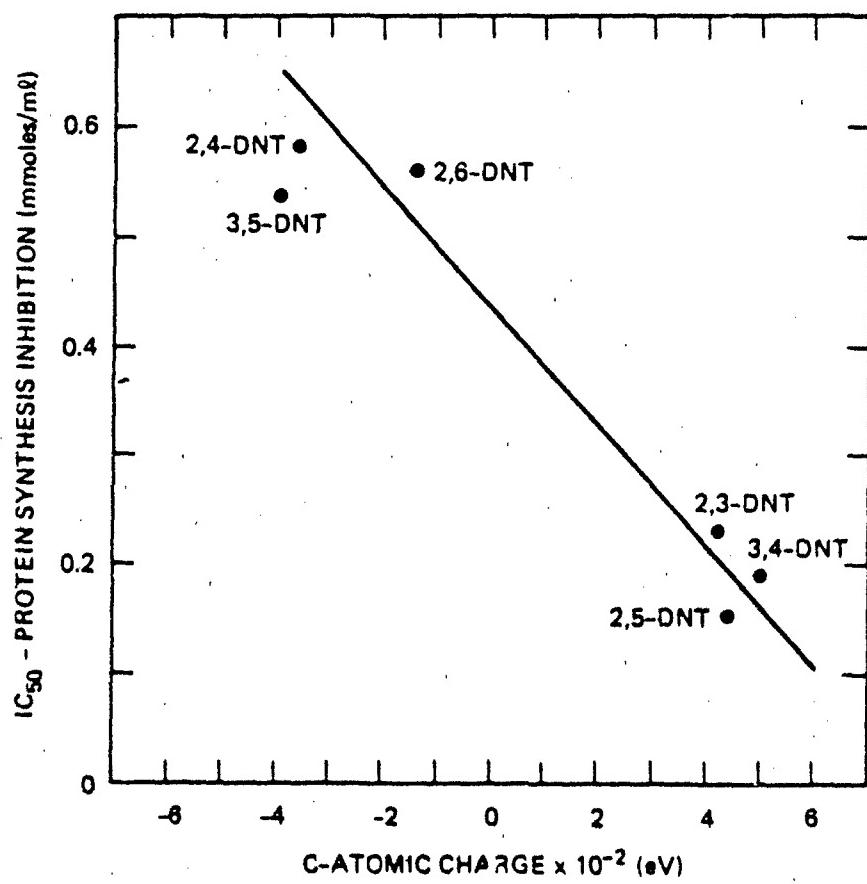
Such correlations as shown in Figures 12 and 13 may be explained by the rate of formation of a DNT-enzyme complex (i.e. Janowsky complex, Pollitt and Saunders, 1965) shown in equation 3.





LA-8251-12

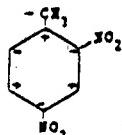
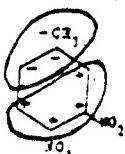
FIGURE 12 LDH RELEASE  $EC_{20}$  VERSUS C-ATOMIC CHARGE



LA-8251-13

FIGURE 13 PROTEIN SYNTHESIS INHIBITION  $IC_{50}$  VERSUS C-ATOMIC CHARGE

This complex formation may be promoted by nucleophilic attack of the enzyme at electrophilic centers in the DNT molecule. This complex is stabilized by resonance delocalization when the nitro groups are oriented ortho or para to each other. Also, the distribution of charge in the ortho-para-oriented DNT isomers tends to polarize the molecule compared to alternating plus-minus charges calculated for the meta-isomers shown below. This may help orient a complex formation to transfer an electron to the DNT molecule without the direct formation of a sigma bond. Thus, not only the magnitude of the charge but also the distribution of charge over the molecule may be critical in complex formation.



From Table 7

On this basis, it should be possible to project an EC<sub>50</sub> value of nitroaromatic compounds with respect to their carbon atomic charge. The atomic charges on carbon atoms bearing nitro groups as computed by MNDO for nitrobenzene, the three isomeric dinitrobenzenes, nitrotoluenes, and isomeric trinitrotoluenes relative to benzene are shown in Table 9.

This table projects several of the trinitrotoluene (TNT) isomers as being extremely cytotoxic if we assume that mechanisms of cytotoxicity for the TNTs are similar to those for the DNTs.

The results of this study did show a difference in slope of the concentration response curve for LDH release (Figure 2) and in chemical metabolism of 2,6-DNT compared with the five other DNT isomers. Oxidation at the methyl group appeared to be the major route of metabolism, probably because of the difficulty in reducing the nitro group due to nonplanarity with the phenyl ring caused by steric crowding.

The formation of a Janovsky-type complex may be the critical step required to eventually produce cytotoxicity (possibly as shown by Equation 3), but this may not be the primary route to hepatocarcinogenicity. 2,6-DNT is the only isomer to have been shown unequivocally to be both hepatocarcinogenic and genotoxic. Our results show methyl group oxidation to be a primary route of metabolism for 2,6-DNT in isolated hepatocytes. If methyl group oxidation is a primary step to carcinogenicity, then it may be possible to relate the charge on the methyl carbon to hepatocarcinogenic potential. On this basis, the DNT isomers would be ranked as shown in Table 10.

**Table 9**  
**CARBON ATOMIC CHARGES AND PROJECTED EC<sub>50</sub> VALUES  
 FOR LDH RELEASE FOR SELECTED NITRO AROMATIC COMPOUNDS**

Compound	<u>C<sub>1</sub></u>	<u>C<sub>2</sub></u>	<u>C<sub>3</sub></u>	<u>C<sub>4</sub></u>	<u>C<sub>5</sub></u>	<u>C<sub>6</sub></u>	Projected EC <sub>50</sub>
Nitrobenzene	-0.034	+0.065	-0.0221	+0.059	-0.027	+0.065	1.6
1,2-Dinitrobenzene	+0.032	<u>+0.032</u>	+0.047	+0.020	+0.020	-0.047	0.42
1,3-Dinitrobenzene	-0.052	+0.134	<u>-0.052</u>	+0.109	-0.043	+0.109	2.3
1,4-Dinitrobenzene	+0.011	+0.045	+0.045	<u>+0.011</u>	+0.045	0.045	0.66
1,3,5-Trinitrobenzene	-0.068	+0.171	-0.068	+0.171	-0.068	+0.171	3.2
2-Nitrotoluene	+0.039	<u>-0.014</u>	+0.057	-0.023	-0.037	-0.019	1.1
3-Nitrotoluene	-0.063	+0.079	<u>-0.036</u>	-0.069	-0.031	+0.056	1.7
4-Nitrotoluene	+0.065	-0.013	+0.062	<u>-0.031</u>	+0.062	-0.013	1.5
2,4,6-Trinitrotoluene	+0.149	<u>-0.047</u>	+0.154	-0.056	+0.154	-0.0471	2.1
2,4,5-Trinitrotoluene	+0.063	+0.008	+0.102	<u>+0.017</u>	<u>+0.065</u>	+0.039	0.22
2,3,5-Trinitrotoluene	+0.008	<u>+0.082</u>	+0.008	+0.111	-0.011	+0.094	<0.1
2,3,4-Trinitrotoluene	+0.058	+0.032	<u>+0.086</u>	+0.020	+0.075	+0.013	<0.1
2,3,6-Trinitrotoluene	+0.096	+0.026	<u>+0.063</u>	+0.032	+0.077	+0.005	0.23
3,4,5-Trinitrotoluene	-0.018	+0.086	+0.019	<u>+0.089</u>	+0.019	+0.087	<0.1

Table 10  
RELATIONSHIP OF METHYL GROUP CHARGE TO CARCINOGENIC POTENTIAL

<u>Compound</u>	<u>Methyl Group Charge</u>	<u>Ranking of Carcinogenic Potential</u>
2,6-DNT	-0.033	1
2,4-DNT	-0.026	2
2,3-DNT	-0.018	3 or 4
2,5-DNT	-0.018	3 or 4
3,4-DNT	-0.011	5
3,5-DNT	-0.003	6

Due to the amount of funds and time provided for this study, our results can only be considered preliminary and have raised many more questions than we are able to answer. Some of these questions are as follows:

- 1) A complete mass balance of parent compound and metabolites needs to be established. This requires that the study be repeated using <sup>14</sup>C-labeled parent compounds and following the distribution of activity by use of radiochemical detector coupled to the HPLC.
- 2) Other methods of detecting free-radical activity, such as electron spin resonance spectroscopy or enzymatic assays (catalase or superoxide dismutase) need to be assessed to confirm that reductive transformation is an integral part of cytotoxicity.

Microsomal and mitochondrial preparations from liver cells allow higher enzyme concentrations in comparison to hepatocytes and could also be used to facilitate detection and identification of nitro anion free radicals in the medium.

3) Does the in vitro rat hepatocyte assay accurately mimic in vivo metabolism? The in vivo metabolism of 2,4-DNT in the rat has been shown to be primarily oxidative (Rickert and Long, 1981), but our in vitro results indicated the contrary. The difference may lie in experimental conditions and protocols which differed greatly from those of Rickert and Long, and limited the production of glucuronide conjugates of oxidative products in the present study.

4) What other theoretical parameters can be used to correlate cytotoxicity and chemical structure? Are LUMO predictions or chemical reduction potentials the proper choices for structure-activity relationships in the case of DNT isomers, or are other factors such as molecular geometries more important?

The biological and chemical behavior of the DNT isomers remains a challenging and stimulating area of research in the development of structure-activity relationships.

If cytotoxicity is a component feature in the promoting activity of carcinogens, as has been proposed in the resistant hepatocyte model for hepatocarcinogenesis (Farber, 1984), the one finding that is clear from the present studies is that ortho-para-substituted DNTs are more potent cytotoxins than meta-substituted DNTs. Consequently, assuming the relationship between cytotoxic and promoting potential applies for DNTs, which appear to exhibit greater activity as promoters than as initiators of hepatocarcinogenesis (Popp and Leonard, 1982), ortho-para-substituted DNTs should be more active promoting agents than the meta-substituted ones that have been tested to date. These analogs may contribute to the activity of technical grade DNT in addition to 2,6-DNT, but this hypothesis requires confirmation from in vivo experiments. We hope that this report will promote further research in this area.

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